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## Functional dissection of the Oct6 Schwann cell enhancer reveals an essential role for dimeric Sox10 binding

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### Abstract

The POU domain transcription factor Pou3f1 (Oct6/Scip/Tst1) initiates the transition from ensheathing, promyelinating Schwann cells to myelinating cells. Axonal and other extra-cellular signals regulate Oct6 expression through the Oct6 Schwann cell enhancer (SCE), which is both required and sufficient to drive all aspects of Oct6 expression in Schwann cells. Thus, the Oct6 SCE is pivotal in the gene regulatory network that governs the onset of myelin formation in Schwann cells and provides a link between myelin promoting signalling and activation of a myelin related transcriptional network. In this study we define the relevant cis-acting elements within the SCE and identify the transcription factors that mediate Oct6 regulation. On the basis of phylogenetic comparisons and functional *in vivo* assays we identify a number of highly conserved core elements within the mouse SCE. We show that core element 1 is absolutely required for full enhancer function and that it contains closely-spaced inverted binding sites for Sox proteins. For the first time *in vivo*, the dimeric Sox10 binding to this element is shown to be essential for enhancer activity whereas monomeric Sox10 binding is non-functional. As Oct6 and Sox10 synergize to activate the expression of the major myelin-related transcription factor Krox20, we propose that Sox10 dependent activation of Oct6 defines a feed-forward regulatory module that serves to time and amplify the onset of myelination in the peripheral nervous system.

### Keywords

Schwann cell; pou3f1; enhancer; transcription; development

### Introduction

Within the peripheral nervous system (PNS), Schwann cells generate and maintain a multi-lamellar insulating myelin sheath around an associated axon and impose cellular specializations that allow fast conduction of action potentials. The generation of myelinating Schwann cells from an embryonic pool of precursors and the elaboration of the myelin sheath is primarily controlled by the axons with which the cells associate (Jessen and

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Mirsky, 2005). Axonal signals that include Neuregulin1, Notch1 ligands and neurotrophins, activate a range of signalling pathways that converge on the nucleus to initiate a program of myelin-related gene transcription (Svaren and Meijer, 2008; Taveggia et al., 2010).

Several Schwann cell autonomous regulators of myelination have been identified and studied over the last decades. These include the transcription factors Sox10, NfκB, Notch, Nfatc4, YY1, Srebp, Oct6, Brn2 and Krox20 (Svaren and Meijer, 2008; Verheijen et al., 2009; He et al., 2010; Taveggia et al., 2010). Perhaps the most intensely studied are the POU-domain transcription factor Oct6 (Pou3f1), the Sry-box protein Sox10 and the zinc finger transcription factor Krox20 (Egr2) (Topilko et al., 1994; Bermingham et al., 1996; Jaegle et al., 1996; Britsch et al., 2001). Whereas Sox10 is expressed at high levels at all stages of the Schwann cell lineage, both Oct6 and Krox20 are expressed dynamically in the Schwann cell lineage during development and nerve regeneration and their expression is highest in actively myelinating cells (Zorick et al., 1996; Arroyo et al., 1998; Kuhlbrodt et al., 1998; Finzsch et al., 2010). Genetic studies have shown that deletion of Oct6 and its close relative Brn2 (also expressed in the Schwann cell lineage with kinetics similar to that of Oct6), causes severe but transient arrest at the promyelin stage of cell differentiation resulting in late onset of myelination and hypomyelinated nerves in adult animals (Bermingham et al., 1996; Jaegle et al., 1996; Jaegle et al., 2003). Deletion of Krox20 similarly results in a differentiation arrest at the promyelin stage but this arrest is insurmountable (Topilko et al., 1994). Data showing that Oct6/Brn2 cooperate with Sox10 to upregulate Krox20 in myelinating cells through its 'myelinating Schwann cell element' (MSE) suggest a model in which Oct6/Brn2 levels serve to time the onset of myelination in response to promyelinating signals and through activation of Krox20 gene expression (Ghislain and Charnay, 2006; Svaren and Meijer, 2008; Reiprich et al., 2010). Thus, insight into how Oct6 itself is regulated is pivotal for our understanding of regulation of myelination in the PNS.

We have previously shown that a 4,3 kb Schwann cell enhancer (SCE) sequence approximately 10 kb downstream of the Oct6 transcriptional start site is required and sufficient to drive temporally correct expression from a linked promoter in Schwann cells (Mandemakers et al., 2000; Ghazvini et al., 2002). To begin to understand the nature of the signalling routes that converge on the SCE we set out to identify the functional elements within the enhancer and identify proteins that bind to these elements.

## Materials and Methods

### Computational analysis of the Oct6 Schwann cell enhancer

Conserved elements within the Oct6 SCE of human, mouse, rat, opossum, wallaby and zebrafish were identified using the VISTA phylogenetic alignment tool (<http://pipeline.lbl.gov/cgi-bin/gateway2>) (Frazer et al., 2004). All sequences were aligned to the mouse sequence. The homology cut off standard is 50% but was adjusted to 90% for the rat sequence because of the close evolutionary relationship between mouse and rat. Likewise, the homology cut off was adjusted to 30 percent when mouse sequences were aligned to opossum, wallaby and zebrafish sequences. Alignments were optimized manually. Aligned sequences were further analyzed for the presence of putative transcription factor DNA binding sites using ConSite: a web-based tool for finding cis-regulatory elements in genomic sequences (Sandelin et al., 2004) (<http://www.phylofoot.org/concite>).

### Cell culture, transient transfection and nuclear extract preparation

Primary Rat Schwann cell cultures were established as described previously (Brockes et al., 1979; Mandemakers et al., 2000). Rat Schwann cells were maintained in Dulbecco's

modified Eagle's medium (DMEM; Lonza), 3% fetal calf serum (FCS; Harlan), 2 $\mu$ M Forskolin (Sigma), 1% Penicillin and Streptomycin (Invitrogen) and 5% NDF- $\beta$  conditioned medium (Spiegel and Peles, 2009) at 37°C with 5% CO<sub>2</sub>. Schwann cells were differentiated *in vitro* towards an early myelinating stage (Morgan et al., 1991), a stage at which they express relatively high levels of Oct6. First, medium was switched to a serum free formulation of DMEM/F12 (Lonza), 1X N2 supplement (Invitrogen), 10ng/ml NGF (Harlan), 0.01% BSA (Invitrogen), 5% NDF- $\beta$  conditioned medium and 1% PS for 18 hours. Cells acquire a spindle shaped form under serum free conditions. Then, differentiation was initiated by addition of 20 $\mu$ M Forskolin (Sigma) or 100  $\mu$ M CTP-cAMP (Sigma). Cells were allowed to differentiate for 36 hours before further analysis. Differentiated cells have flattened cell morphology and express high levels of Oct6.

RT4-D6P2T rat Schwannoma cells (Bansal and Pfeiffer, 1987; Gandelman et al., 1989) were obtained from ECACC and were maintained in DMEM, 10% FCS and 1% PS. For nuclear extract preparation RT4-D6P2T cells were grown in 15cm dishes to near confluence. When confluent, cells were washed once with PBS, scraped in cold PBS and nuclear extracts were prepared essentially according to Dignam and colleagues (Dignam et al., 1983). Nuclei were extracted in 20 mM Hepes-KOH pH7.6, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5% NP-40, 0.5 mM DTT and 1 x complete protease inhibitor mix (Roche). The salt concentration of the extract was reduced by dialysis against a buffer containing 20 mM Hepes-KOH pH7.6, 25% glycerol, 100 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 95  $\mu$ g/ml sodium metabisulfite. Precipitates were removed by centrifugation two times at 13,000 rpm for 15 minutes, at 4°C. The dialyzed nuclear extract was snap-frozen and stored in aliquots at -80°C.

HEK293T cells were maintained in DMEM, 10%FCS and 1% PS. HEK293T Cells were transfected with 20 $\mu$ g pCMV promoter driven full length Sox10 or MIC Sox10 (kind gift from Prof. Michael Wegner) expression cassettes in 10 cm dishes using the polyethylenimine (PEI) method. Cells were harvested 48 hours post-transfection for nuclear extract preparation (Jaegle et al., 2003).

### Luciferase assay

Rat Schwann cells were seeded in 6 well Primaria dishes (Becton Dickinson) and grown to 70–80% confluence. Cells were transfected in triplicate with 1.125 $\mu$ g pGL3 luciferase reporter plasmid (Promega) containing different SCE fragments and 0.375 $\mu$ g pCMV- $\beta$ gal reference expression plasmid in the presence of FCS, using FUGENE6 transfection reagent (Roche) at a 1:4 (DNA:FUGENE6) ratio. Cells were washed 18 hours post transfection, and medium was switched to a serum free formulation (DMEM/F12 (Lonza), 1X N2 supplement (Invitrogen), 10ng/ml NGF (Harlan), 0.01% BSA (Invitrogen), 5% NDF- $\beta$  conditioned medium and 1% PS). After overnight incubation in this medium, cells were induced to differentiate through addition of CTP-cAMP to a final concentration of 100 $\mu$ M, and cultured for an additional 36 hours. Cells were harvested 72 hours post transfection, washed and lysed in 1X lysis buffer (Promega) and extracts were assayed for luciferase activity using Steady GLO luciferase substrate buffer (Promega) and  $\beta$ -galactosidase activity using 2-nitro-phenyl-galacto-pyranoside (ONPG) substrate. Luciferase activities were normalized for  $\beta$ -galactosidase activity. Both luciferase and  $\beta$ -galactosidase levels were measured on a luminometer (Perkin Elmer – Victor3). All the experiments were performed at least three times in triplicate.

## DNA cloning and SCE deletion constructs

The mouse SCE was originally cloned as an HpaI-MscI DNA fragment from a mouse 129sv cosmid library (Mandemakers et al., 2000). This fragment, which corresponds to position 124,342,041–124,349,427 on mouse chromosome 4 of genome build37 (NCBIM37), was used to generate, by PCR, a series of 10 deletion constructs in which a shifting window of approximately 500 basepairs is deleted from the entire SCE. SalI and BglII restriction sites were introduced at the 5' and 3' end respectively and these sites were used to clone the fragments into the SV40 promoter driven pGL3 luciferase reporter plasmid. Subfragments from the SCE such as HR1a and HR2 were generated by PCR using the HpaI-MscI SCE fragment as template and cloned into luciferase and LacZ reporter plasmids (Mandemakers et al., 2000). Fragment HR1a corresponds to position 124,344,894–124,345,418 and HR2 to position 124,347,550–124,348,429 on mouse chromosome 4 (NCBIM37).

## Transgenic mice and whole mount $\beta$ -galactosidase staining

Transgenesis and identification of transgenic embryos by PCR and southern blotting was performed as described previously (Mandemakers et al., 2000). LacZ expression was visualized by whole-mount X-gal staining of P2-P4 pups or nerves dissected from transgenic animals at different stages of postnatal development. In brief, young mice (2–4 days of age and of either sex) were anaesthetized and sacrificed by decapitation. The body cavity was opened and the major organs were removed. All of the tissue was transferred to a solution of fixative containing 1% formaldehyde, 0.2% Glutaraldehyde, 2mM MgCl<sub>2</sub>, 5mM EGTA, 0.02% NP40 in PBS, for 1 hour at room temperature (RT). Tissues were washed in PBS/0.02% NP40 for 3 times at RT.  $\beta$ -galactosidase staining was carried overnight at RT in staining solution containing 5mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 5mM K<sub>4</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O, 2mM MgCl<sub>2</sub>, 0.01% Sodium deoxycholate, 0.02% NP40 and 1mg/ml 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal). The staining was stopped by extensively washing in PBS/0.02% NP40 and post fixed in a 4% formaldehyde fixative.

All animal experiments were performed according to guidelines and protocols that had been approved by an independent committee on the ethical use of experimental animals (DEC).

## Electrophoretic mobility shift assay (EMSA)

Equal amounts of nuclear extract were used in the EMSA using 50 fmole of a <sup>32</sup>P end-labeled double-strand oligonucleotide probe. Probe and nuclear extracts were incubated on ice, for 30 minutes in 10mM HEPES-KOH pH 7.9, 100 mM KCl, 5mM MgCl<sub>2</sub>, 0.1% EDTA, 2mM DTT, 4 $\mu$ g BSA and 4% Ficoll in the presence of 2 $\mu$ g of competitor DNA, poly dG-dC (Sigma). Total reaction volume was 20 $\mu$ l. In super shift experiments, reaction mixtures were further incubated for 20 minutes with 1 $\mu$ g of Goat-Sox10 antibody (Santacruz). Complexed and free probe were separated on a 4% polyacrylamide gel which was pre-run for 30 minutes at 120 volts in 0.5X TBE electrophoresis buffer at RT. Gels were fixed in 10% methanol/10% acetic acid for 20 minutes, dried and exposed to a Phospho-imaging screen (Molecular dynamics). ImageJ software was used to analyze the band intensities. Probes used in the band shift assays are shown in Figure 5.

## Chromatin immunoprecipitations

For Chromatin immunoprecipitation (ChIP) assays in RT4D6 cells (20x10<sup>6</sup> cells/IP), dual cross-linking was performed using di(N-succinimidyl)glutarate (DSG) and formaldehyde (van den Berg et al., 2008). Cells were washed four times with PBS, followed by incubating the cells with cross linking agent, 2mM DSG on a rotating platform at a medium speed for 45 minutes at RT. After washing the cells four times with PBS, conventional formaldehyde cross linking was performed by incubating the cells in freshly prepared buffered



formaldehyde (50mM Hepes KOH pH 7.5, 100mM NaCl, 1mM EDTA, 0.5mM EGTA, 1% formaldehyde) for 10 minutes. 2.5M of Glycine was added to a final concentration of 100mM to quench the formaldehyde and cells were further incubated for 5 minutes at RT. From here on all the subsequent steps were carried at 4°C in the presence of complete EDTA free protease inhibitor (Roche). Cells were scraped in cold PBS and spun at 2000 rpm for 5 minutes. Cell pellet was resuspended in SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris pH 8.0) and subsequently sonicated for 15 cycles with 15-s on and 30-s off regime. The sheared chromatin was cleared of insoluble debris by centrifugation at 13000 rpm for 10 minutes. The supernatant containing the sheared chromatin was pre-cleared with protein A Agarose/Salmon sperm DNA (Millipore) for one hour at 4°C. 1% chromatin was set aside to serve as a 1/100<sup>th</sup> of total chromatin input control. After centrifugation, the supernatant was incubated with 10 µg of anti-Sox10 antibody (Abcam) or 10 µg of normal rabbit immunoglobulin G (IgG; Santa Cruz Biotechnology) control antibody at 4°C on a rotating wheel overnight. Immune complexes were incubated with 60 µl of protein A Agarose/Salmon sperm DNA slurry for one hour at 4°C. After centrifugation, the immune complexes were washed once with 1 ml of low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.0, 150mM NaCl), once with high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.0, 500mM NaCl), once with Lithium chloride wash buffer (0.25M LiCl, 1% NP-40, 1% deoxycholic acid(sodium salt), 1mM EDTA, 10mM Tris pH 8.0), and twice with TE buffer (10mM Tris pH8.0, 1mM EDTA) (all the wash buffers contain protease inhibitor). Immunoprecipitated chromatin was eluted twice each time with 250 µl of elution buffer (1% SDS, 0.1M NaHCO<sub>3</sub>). The elutions were pooled and 5M NaCl was added to a final concentration of 300mM. Further, cross-links were reversed by incubating the sample at 65°C for 5 hours. The solution was adjusted to 10mM EDTA and 10mM Tris-HCl pH 6.5. ProteinaseK (10mg/ml) was added to 40µg/ml and the solution was incubated for one hour at 45°C. DNA was purified with phenol:chloroform:isoamyl alcohol, precipitated with EtOH and dissolved in water. The same reverse cross-linking and purification procedure was followed for the input sample. Comparative Ct values (Livak and Schmittgen, 2001) were used to calculate the enrichment of a DNA segment relative to the total input. These values were used to calculate the relative enrichment of Sox10 antibody over rabbit IgG precipitated chromatin for the c1, -13kb and IgG2a promoter regions. Primer sets used in quantitative PCR for ChIP are as follows: **C1 of SCE:** 5'-GCC CTG AGG ACT CTA GCT CT-3' and 5'-GCA GGA CAA TAG CTG CAT TC-3', **-13kb:** 5'-AAT CAC GTC CTC ACC AAA CC-3' and 5'-TCC GGT TAG CTC GAA TCC TA-3', **IgG2aP:** 5'-GAA ATT CTG CCC TGC ACT TCC-3' and 5'-GCT TTG CAT TGA GGG AGG ATC-3'

### In vivo formaldehyde assisted identification of regulatory elements (FAIRE)

Freshly isolated P14 rat sciatic nerves were minced in phosphate buffered saline (PBS) with 1% formaldehyde for 5 minute at RT. The nerves were washed with cold PBS and frozen at -80°C. Following thawing, the nerves were analyzed by FAIRE as previously described (Giresi and Lieb, 2009; Jang et al., 2010). A custom microarray (Nimblegen Roche) consisting of 50kb Oct6 rat gene locus was used for identification of regions enriched by FAIRE. Labeling of the FAIRE with Cy5 and total input with Cy3 followed by micro-array hybridization was performed as described (Jang and Svaren, 2009) by Nimblegen. The enrichment ratio of Cy5 to Cy3 was plotted on a Log<sub>2</sub> scale and further processed to display a moving average using a window size of 5 probes. All raw data sets for the custom tiled array are available from the NCBI Gene Expression Omnibus website: accession number GSE23648.

## Results

### The Oct6 SCE contains two functionally interdependent conserved modules

Previously, DNaseI hypersensitivity mapping and alignment of the genomes of human, mouse and rat using the VISTA alignment tool presented here, localized several possible Oct6 regulatory regions including the SCE (Figure 1A). Among the strongly conserved regions within the Oct6 locus, only three regions (at -8, -0.5 and +10.5kb) correspond with previously identified DNaseI hypersensitive sites (HSS; Figure 1A; numbered arrows). The conserved peak at -4.8kb corresponds with an estradiol responsive enhancer characterized by Renner and colleagues (Renner et al., 1996). The large conserved region containing the Oct6 gene contains the promoter proximal region at -0.5kb which confers Interferon- $\beta$  responsiveness and is important for the regulated expression of Oct6 in macrophages (Hofmann et al., 2010). An ultraconserved element flanks the 3' end of the locus (Figure 1A; arrowhead) at approximately +15kb (Bejerano et al., 2004) and is downstream of the SCE. All of these elements are dispensable for Oct6 expression *in vivo* in the Schwann cell lineage. The relevance of the other conserved elements, outside the boundaries of the SCE, for the regulated expression of Oct6 in cells other than Schwann cells is not known.

Within the defined boundaries of the SCE there are two pairs of conserved elements, which we termed HR1a and HR1b (at +10 to 10.8kb) and HR2a and HR2b (at +13 to 13.7kb). The HR1 region corresponds to DNaseI hypersensitive site 6, whereas HSS7 maps to a marginally conserved region between HR1 and HR2. To test the relative contributions of conserved elements within the enhancer to Schwann cell specific expression in transgenic mice we generated a number of constructs that were linked to the generic minimal promoter of the Hsp68 gene driving expression of the LacZ gene (Figure 1B). These constructs were analysed for Schwann cell specific expression of the LacZ reporter in the sciatic nerve of F<sub>0</sub> transgenic animals at postnatal day 2 to 4 by whole mount X-gal staining. The blue sciatic nerve in Figure 1B is a typical example of such analysis. Confirming our previous results (Mandemakers et al., 2000), the full 4.3 kb SCE construct confers Schwann cell specific expression in transgenic animals with high frequency (construct 1 Figure 1B; 20 out of 26 transgenic animals), whereas the 5' region (construct 2; 1 out of 7) and 3' region (construct 3; 0 out of 4) alone did not drive expression in Schwann cells at an appreciable frequency. However, these constructs abutted in the HSS7 region leaving open the possibility that in both these constructs essential genetic information was disrupted. To examine this possibility 5 constructs (construct 4 to 8) spanning the width of the SCE were generated. Only one (construct 6) of these constructs conferred Schwann cell expression on the hsp68 promoter in transgenic mice although again at a low frequency (1 out of 6 transgenic animals). We then combined DNA fragments covering HR1 or HR1a and HR2 in one construct (construct 9 and 10 respectively) and found that both fragments are required to confer high frequency of reporter gene expression in Schwann cells of transgenic mice. Furthermore, these data show that HR1 can be reduced to HR1a without loss of activity.

Active enhancer elements are associated with a relatively open chromatin configuration, devoid of nucleosomes, and these can be probed using FAIRE (formaldehyde assisted isolation of regulatory elements) (Giresi et al., 2007). Using this assay on chromatin derived from two week old rat sciatic nerve, strong peaks at both HR1a and HR2 were found, in line with our functional transgenic expression results. Thus, both regions are associated with a relatively open chromatin structure and are interdependently required for formation of an active enhancer in Schwann cells.

## Deletion analysis of the SCE in cultured Schwann cells

To more finely delineate the essential elements within the Schwann cell enhancer an *in vitro* rat Schwann cell culture system was used, since it is rapid and inexpensive compared to transgenic mouse experimentation. Primary rat Schwann cells can be cultured indefinitely (Brockes et al., 1979; Mathon et al., 2001) and, when exposed to agents that elevate intracellular cAMP levels, can be induced to differentiate into a cell that resembles an early myelinating Schwann cell. Under these conditions endogenous Oct6 expression is induced within three hours reaching maximum levels after 24 hours (Monuki et al., 1989). We generated a series of deletion constructs ( $\Delta 1$  to  $\Delta 10$ ) covering the entire SCE (and cloned these in a SV40 promoter driven luciferase vector; Figure 2A and 2B). These constructs were co-transfected with a CMV promoter driven  $\beta$ -galactosidase reference reporter into rat Schwann cells. Cells were cultured for 18 hours in serum-free medium, switched to differentiation medium for another 36 hrs and measured for luciferase and  $\beta$ -galactosidase activity. All luciferase values were normalized for  $\beta$ -galactosidase activity. The activity of the entire enhancer was set to 100% (Figure 2B) and activity of the various SCE deletions is reported as percentage of the full SCE activity. From this deletion screen it is evident that a major enhancing activity is associated with the region covered by  $\Delta 3$  and, to lesser extent,  $\Delta 2$  and  $\Delta 10$ . These regions coincide with the HR1 and HR2 conserved regions.

Next we tested whether the HR1 and HR2 regions, alone or in combination, would enhance gene expression in transfected Schwann cells (Figure 2B; constructs  $\Delta 11$ ,  $\Delta 12$  and  $\Delta 13$ ). In contrast with our transgenic data, we found that HR1 ( $\Delta 12$ ) is as active as the full enhancer although this activity is further enhanced by the presence of HR2 (as in  $\Delta 11$ ). HR2 by itself is less active than the full enhancer. Further restriction of HR1 to HR1a as in construct  $\Delta 14$  and  $\Delta 15$  suggested that the major enhancer activity of HR1 is located within HR1a, but that this activity is boosted by the presence of HR2 sequences.

To identify the major functional elements within the HR1a region we tested a series of smaller overlapping fragments HR1a1 through HR1a5 (Figure 2B; constructs  $\Delta 16$  to  $\Delta 21$ ). Whereas  $\Delta 17$  and  $\Delta 18$  gave full enhancer activity as compared to control  $\Delta 16$  (HR1a),  $\Delta 19$  further enhanced activity by 5-fold. Constructs  $\Delta 20$  and  $\Delta 21$  showed reduced activity. Interestingly, the HR1a3 sequence associated with very high enhancer activity contains several consensus binding sites for transcription factors known to be involved in Schwann or neural crest cell biology (Figure 2C). These include binding sites for Sox, Snail and Creb/AP-1 proteins, indicating a likely important role for these proteins in mediating the enhancer effect in differentiating Schwann cells.

## A 35 bp core element is essential for Oct6 activation in Schwann cells in vivo

The activity of HR1a3 in combination with HR2 was tested in transgenic mice. Surprisingly, we found that HR1a3 has no activity *in vivo* (Figure 3A; construct 12). No Schwann cells were found to express LacZ in any of the 11 transgenics tested. Moreover, an additional construct that covers all of HR1a3 and extends to HR2 and 3' beyond the boundaries of HR2 (construct 11) also did not show activity *in vivo*. These results are in stark contrast with the activity of construct 10, which contains 175bp of extra sequence 5' to HR1a3 (see Figure 1B), and demonstrate that an essential element is missing in constructs 11 and 12.

To identify the missing element(s) we extended our phylogenetic analysis of the SCE to include the genomes of the Opossum (a non-placental mammal) and the Zebrafish. As is evident in Figure 3B, homology among the orthologous sequences rapidly breaks down when more distantly related species are included. Note that the threshold for the genomes of Opossum and Zebrafish has been lowered to 30%. However, when we manually aligned the sequences abutted by the 5' border of construct 10 and 11, one short element of



approximately 35 basepairs was found strongly conserved in mammals and marsupials (Figure 3C). We refer to this short conserved element as core 1 or c1.

To test whether this c1 sequence represents an essential element of the SCE, we cloned the sequence in front of HR1a3 of construct 12 to generate construct 13. Indeed, this construct drives Schwann cell specific expression of the reporter in 9 out of 18 transgenic mice (Figure 3A), demonstrating that this short element contains genetic information that is essential for Schwann cell specific enhancer function *in vivo*.

### Temporal control of gene expression through the SCE is promoter independent

Previously, our experiments showing that the Oct6 SCE drives temporally correct expression of a reporter gene in the Schwann cell lineage, including its downregulation in fully differentiated cells, were performed with the Oct6 promoter driving reporter gene expression (Mandemakers et al., 2000). The use of the Oct6 promoter leaves open the possibility that the observed down regulation of the reporter gene was mediated through Oct6 promoter sequences and/or the SCE. Now, to test whether SCE construct 13 contains all the information to drive regulated expression and does so irrespective of the linked promoter, transgenic lines (two lines for each construct) were generated in which the LacZ reporter is driven by the Oct6 promoter or the Hsp68 promoter. We choose one line for each construct with comparable copy-number for further analysis (Figure 4A). LacZ reporter gene expression in the sciatic nerve of transgenic mice was monitored at different stages of development and compared with LacZ expression in nerves of mice carrying a LacZ-neo fusion ( $\beta$ geo) gene knocked in the Oct6 locus (Jaegle et al., 1996). As shown in Figure 4B, SCE construct 13 drives temporal expression from both the Oct6 and Hsp68 promoter in a pattern that largely follows that of the endogenous Oct6 gene (Oct6 $\beta$ geo). Both lines show up-regulation of reporter gene expression at later stages of embryonic development (E16 to first day of postnatal life) and expression is down-regulated after the first week of postnatal development and extinguished at later stages. However, expression of Hsp68LacZSCE13 is initiated slightly ahead of Oct6 $\beta$ geo, but is extinguished correctly, whereas Oct6LacZSCE13 expression is initiated correctly but is extinguished with slower kinetics. As we analysed only one line for each construct we do not know whether these marginal deviations in expression are caused by chromosomal integration position effects or whether they reflect differences between the Oct6 and Hsp68 promoter. Nevertheless, it is clear that SCE construct 13 contains all elements to respond to the transcription factor environment of differentiating Schwann cells and the signalling pathways that regulates their activities and that no Oct6 promoter specific sequences are required for downregulating Oct6 expression in mature myelin forming cells.

### Core element 1 contains an atypical dimeric binding site for Sox10

How does core element 1 (c1) contribute to Schwann cell specific enhancer function of the SCE? Using online bioinformatic tools we identified potential binding sites within this 35bp sequence for several classes of transcription factors including Sox proteins. Sox10, Sox2, Sox13 and Sox4 (Kuhlbrodt et al., 1998); and data not shown) are expressed in immature Schwann cells. Whereas all of these proteins bind to the consensus Sox binding site (A/T) (A/T)CAA(A/T)G, as found in element HR1a3, only Sox10 and Sox13 (not shown) bind to the c1 element in electrophoretic mobility shift assays (EMSA) (Figure 5A; (Harley et al., 1994). We examined the formation of protein-DNA complexes on the c1 probe using rat Schwannoma RT4D6 nuclear extracts and nuclear extracts of HEK293 cells transfected with a Sox10 expression cassette (Sox10 in Figure 5A). RT4D6 cells resemble an early myelinating cell as it expresses considerable amounts of Oct6, Krox20, Sox10 and low amounts of Mpz (Hai et al., 2002; Lindsley et al., 2007); and unpublished observations) and thus provide an excellent model system to study promyelination-related transcription. Three

major complexes were observed (indicated with asterisks in Figure 5A) of which the slowest migrating complex contains Sox10 as evidenced by the further decrease in electrophoretic mobility of this complex after addition of Sox10 specific antibodies (Figure 5A). Interestingly, the Sox10 containing complex on the c1 probe has a lower mobility than the Sox10 complex formed on the c2 probe (Figure 5A) suggesting that Sox10 binds to c1 as a homo- or heterodimer. The sequence of the c2 probe (Figure 5E) is derived from fragment HR1a3 and contains a consensus Sox binding site (underlined in Figure 2C). Homodimeric binding of Sox10 has been described before and depends on the presence of two closely spaced heptameric Sox binding sites in an inverted orientation (Peirano and Wegner, 2000). Examination of the c1 sequence revealed the presence of a degenerate Sox binding site (AACTGAG) in opposite orientation and 4 basepair spacing with the degenerate CACAAAC Sox binding site (Figure 5E). To confirm that Sox10 binds to c1 as a dimer we performed EMSA (Figure 5B) with nuclear extracts of HEK293 cells transfected with an expression construct encoding a truncated form of Sox10 (Sox10 MIC; aminoacid 1–189 including the Sry-box). It has been shown before that this shorter form of Sox10 binds predominantly as a dimer on closely spaced inverted Sox binding sites such as present in the Mpz promoter (C/C' Figure 5E; see (Peirano and Wegner, 2000). Indeed, Sox10MIC binds as a dimer to c1 and this binding is abolished when the 5' Sox site is mutated (c1<sup>mut</sup>). Thus, the two degenerate Sox binding sites bind Sox10 strongly as a dimer whereas the individual 3' Sox site has very little affinity for Sox10.

Next we examined whether indeed Sox10 binds to the c1 SCE motif in chromatin of rat Schwannoma RT4D6 cells. We performed chromatin immune-precipitation assay (CHIP) using an antibody against Sox10 and control rabbit IgGs. We found c1 SCE sequences precipitated with three-fold higher efficiency with Sox10 antibodies versus control IgG antibodies. Two control sequences, a –13kb sequence upstream of Oct6 and the IgG2a promoter showed no enrichment for Sox10 and were precipitated with equal efficiency by Sox10 and IgG antibodies (Figure 5C). Thus, Sox10 occupies c1 SCE chromatin in RT4D6 cells.

To test whether this dimeric Sox10 binding contributes to SCE function *in vivo* we generated a number of reporter constructs and tested their activity in transgenic animals. All these constructs are based on construct 12 and include HR1a3 and HR2. First we tested whether mutation of the 5' degenerate Sox site had any effect on the contribution of c1 to SCE activity. Mutating 5 nucleotides, including the particularly important central CAA motif, as in c1<sup>mut</sup> (Figure 5E) results in loss of Sox10 binding (Figure 5B). Replacing the c1 sequence in construct 13 for the c1<sup>mut</sup> sequence, to generate construct 14, results in a strong reduction of the number of transgenic mice expressing the LacZ reporter in Schwann cells of the sciatic nerve, from 9 out of 18 to 1 out of 9 (Figure 5D). Thus, Sox10 binding to c1 is an essential component of the fully active SCE.

As c1<sup>mut</sup> completely abolishes Sox10 binding we could not determine whether dimeric Sox10 binding per se, or perhaps monomeric Sox10 binding, would suffice for SCE function. To answer these questions we tested two additional constructs. In the first construct tested, construct 15, we replaced the c1 sequence with that of the well documented C/C' dimeric Sox10 binding site from the Mpz promoter (Peirano and Wegner, 2000). In the second construct tested, construct 16, we replaced the c1 sequence for the C/C' derivative C, to which Sox10 binds exclusively as a monomer (sequence is shown in Figure 5E). As is evident from the numbers presented in Figure 5D, the Mpz promoter derived C/C' Sox binding site is as effective as the original c1 sequence in supporting Schwann cell specific expression (construct 15; 7 out of 10). However, the monomeric Sox site C is not an effective substitute for c1 (Figure 5D construct 16; 1 out of 6). Thus, these experiments show that it is dimeric binding of Sox10 to the c1 sequence and not just Sox10 binding per

se that provides an essential function in Schwann cell specific regulation through the Oct6 SCE.

## Discussion

We have combined comparative genomics and transgenic analysis to identify regulatory regions within the Oct6 SCE that are required to drive Schwann cell specific expression in transgenic mice. We demonstrate that a 35 bp fragment called c1 is an essential element of the SCE and contains two degenerate Sox protein binding sites in an inverted orientation. Sox10 protein binds to this element as a dimer and we demonstrate that its *in vivo* activity critically depends on dimeric binding of Sox10, as enhancer activity is lost in DNA mutants that bind Sox10 as a monomer. Thus, dimeric Sox10 binding provides a crucial function within the larger context of the Oct6 SCE including HR1a and HR2, allowing the formation of a productive enhanceosome in response to developmental cues.

Phylogenetic comparison of genome sequences is a powerful tool to identify putative gene regulatory sequences. We initially analyzed human, mouse and rat sequences of approximately 50kb surrounding the Oct6 gene and identified several conserved DNA elements surrounding the Oct6 structural gene (Figure 1A). Within the boundaries of the SCE two pairs of highly conserved blocks could be identified. Inclusion of non-placental mammals and fish in our analysis however shows that the high degree of homology is lost over larger evolutionary distances (Figure 3). This rapid decline in overall sequence conservation down the phylogenetic ladder is remarkable and suggests that the core elements of the enhancer are relatively insensitive to the composition and sequence of the DNA in which they are embedded. In line with this observation we found that the 35bp c1 could be lifted from its natural sequence context and placed directly in front of HR1a3 and HR2 to generate a functional enhancer (Figure 3A).

HSS1 is associated with the promoter region of Oct6 and is embedded in a CpG island. Promoter proximal elements mediate interferon and dsRNA responses in fibroblasts, macrophages and possibly Schwann cells through binding of the transcription factor Stat1 (Hofmann et al., 2010). These elements fall within the limits of HSS1. Our analysis of transgenic founder animals carrying different SCE constructs did not reveal a role for promoter proximal sequences in directing Schwann cell specific expression. However, it did not rule out a possible contribution of these sequences to the temporal control of Oct6 gene expression, in particular down-regulation in fully myelinating cells. Oct6 down-regulation in myelinating Schwann cells is important as forced expression of Oct6 beyond the initial stages of myelination results in myelin degeneration and neuropathy (Ryu et al., 2007). It has been suggested that Oct6 gene expression is extinguished through an auto-inhibitory mechanism and/or through the action of Krox20 (see Figure 6) possibly through binding to the many potential Krox20 binding sites in the Oct6 promoter (Jaegle and Meijer, 1998; Zorick et al., 1999; Svaren and Meijer, 2008). Our results with SCE13 containing constructs, shown in Figure 4, demonstrate that the Oct6 promoter can be replaced by the minimal Hsp68 promoter. The downregulation of these constructs in mature nerves suggests that downregulation is mediated through the SCE and does not depend on Oct6 promoter specific sequences. Whether Oct6 down-regulation involves active repression by Krox20 or other factors, or loss of activation is an open question. Further insight into the developmental down-regulation of Oct6 will depend on the future identification of the relevant sequences within the SCE.

Our detailed mapping of HR1a using a transient reporter Luciferase assay defines a 177bp fragment HR1a3 that is highly active in differentiating rat Schwann cells. This element contains binding sites for a number of transcription factors that are expressed in Schwann

cells including Sox, Snail, cAMP-response-element-binding (CREB) and fos/jun proteins (Figure 2). Of particular interest here is the CRE/AP1 binding site TGCCTCA which has been shown to mediate cAMP induced expression of dopamine  $\beta$ -hydroxylase (Swanson et al., 1998). Increased intracellular cAMP concentrations activate PKA which in turn can activate a variety of proteins including CRE-binding protein (CREB) and the p65 subunit of Nf $\kappa$ B (Tabernero et al., 1998; Yoon et al., 2008). A role for cAMP/PKA in Oct6 expression in Schwann cells was long suspected but compelling evidence for such a role was obtained only recently. It was shown by Monk and colleagues that activation of Oct6 in zebrafish Schwann cells depends on the G-protein coupled receptor Gpr126, a receptor hypothesized to signal through adenylyl cyclase (Monk et al., 2009). Indeed, pharmacological activation of adenylyl cyclase in Gpr126 mutant fish larvae activated Oct6 expression in Schwann cells and rescued the myelination defect in these mutant animals. Phosphorylation of CREB was also shown to result from Nrg1 signalling through the ErbB2 and ErbB3 heterodimeric receptor type tyrosine kinases, suggesting that both Gpr126 and ErbB2/ErbB3 signals contribute to regulated Oct6 gene transcription (see Figure 6) possibly through the CRE/AP1 element present in HR1a3.

However, whereas Luciferase reporter constructs carrying the HR1a3 and HR2 fragment were highly active in transiently transfected rat Schwann cells, a LacZ reporter construct carrying the same enhancer fragments was inactive in transgenic mice (Figures 2 and 3). This discrepancy could reflect differences in Oct6 regulation in Schwann cells of different species. Indeed, it has recently been shown that mouse and rat Schwann cells exhibit different sensitivities to differentiation cues *in vitro* (Arthur-Farraj et al., 2011). Alternatively, the observed discrepancy reflects the fundamentally different nature of the two assays employed here. In transfection assays, the DNA construct remains episomal, doesn't incorporate epigenetic marks and is relatively open to the transcription factor environment of the nucleus. In contrast, a transgenic reporter construct is integrated in the chromosome, acquires epigenetic marks and is subject to chromosomal position effects. Clearly, inclusion of the conserved 35bp fragment c1 (Figure 3 and 4) containing two inversely oriented heptameric Sox10 binding sites restored *in vivo* enhancer activity to the HR1a3 and HR2 DNA fragment and thus provides information that allows productive enhancer function in a chromatin context. Interestingly, two monomeric Sox10 sites as in construct 16 (C and the c2 sequence embedded in HR1a3; Figure 5D and E) are no substitute for these inversely oriented sequences in c1 to which Sox10 binds as a dimer. Sequence dependent dimerization is a property shared by all group E Sox proteins and depends on a short peptide sequence in the aminoterminal portion of the proteins (Peirano and Wegner, 2000). The relevance of dimeric SoxE binding first became apparent when it was found that mutations in Sox9 that abolish dimeric binding but not monomeric binding, cause a form of Campomelic dysplasia in humans (Sack et al., 2003). Likewise, mutation of the homologous sequence in mouse Sox10 (Sox10<sup>aa1/aa1</sup>) results in loss of dimeric DNA binding, causing specific defects in neural crest derived cell lineages. These include Schwann cells which are normally generated but fail to enter the promyelinating phase of differentiation and fail to activate Oct6 expression (Schlierf et al., 2002; Schreiner et al., 2007). Our demonstration here that the c1 element is a target for dimeric Sox10 binding and is essential for Oct6 activation provides a clear explanation for that observation.

How could dimeric Sox10 binding contribute to regulated expression of Oct6? A dimeric Sox10 protein binds DNA with higher affinity and induces stronger bending of the DNA helix than a monomeric Sox10 protein (Peirano and Wegner, 2000). Productive enhancer formation by dimeric Sox10 could result from the higher DNA binding affinity or, alternatively, DNA tethered Sox10 dimers assemble protein complexes different from those assembled by Sox10 on monomeric sites. It is in principle possible that the differences in DNA topology induced by monomeric and dimeric Sox binding contribute to the latter. It is

of note that in Sox10<sup>aa1/aa1</sup> mice Schwann cells are generated in normal numbers whereas in the Sox10 null mice Schwann cells are not generated at all (Britsch et al., 2001; Schreiner et al., 2007). As Sox10<sup>aa1</sup> proteins can still bind as a monomer to Sox binding sites, this suggests that activity of a subset of Sox10 target genes in the Schwann cell lineage depends on monomeric Sox10 binding, whereas another subset of genes including Oct6 depends on dimeric Sox10 binding. Furthermore, our transgenic data suggest that dimeric Sox10 creates an open chromatin configuration in Schwann cells, allowing productive interactions of transcription factors with the Oct6 SCE. Irrespective of whether this results from qualitative or quantitative differences between the two modes of Sox10 binding, the functional implications are profound.

As Sox10 is expressed at high levels at all stages of Schwann cell differentiation, the dependency of the Oct6 SCE on Sox10 does not provide a ready explanation for the dynamic expression of Oct6 during Schwann cell differentiation. Most likely this information is encoded in HR1a3 or HR2. However, we cannot rule out the possibility that the activity of Sox10 on the Oct6 SCE is regulated through modifications such as sumoylation (Taylor and Labonne, 2005; Girard and Goossens, 2006). Indeed, it has been shown that sumoylation of Sox10 represses its capacity to activate specific promoters such as those of the Mitf and Gjb genes (Girard and Goossens, 2006).

It is expected that future identification of additional transcription factors interacting with regulatory elements within HR1a3 and HR2 will provide further insight into the temporal regulation of Oct6 gene expression and myelin related gene expression.

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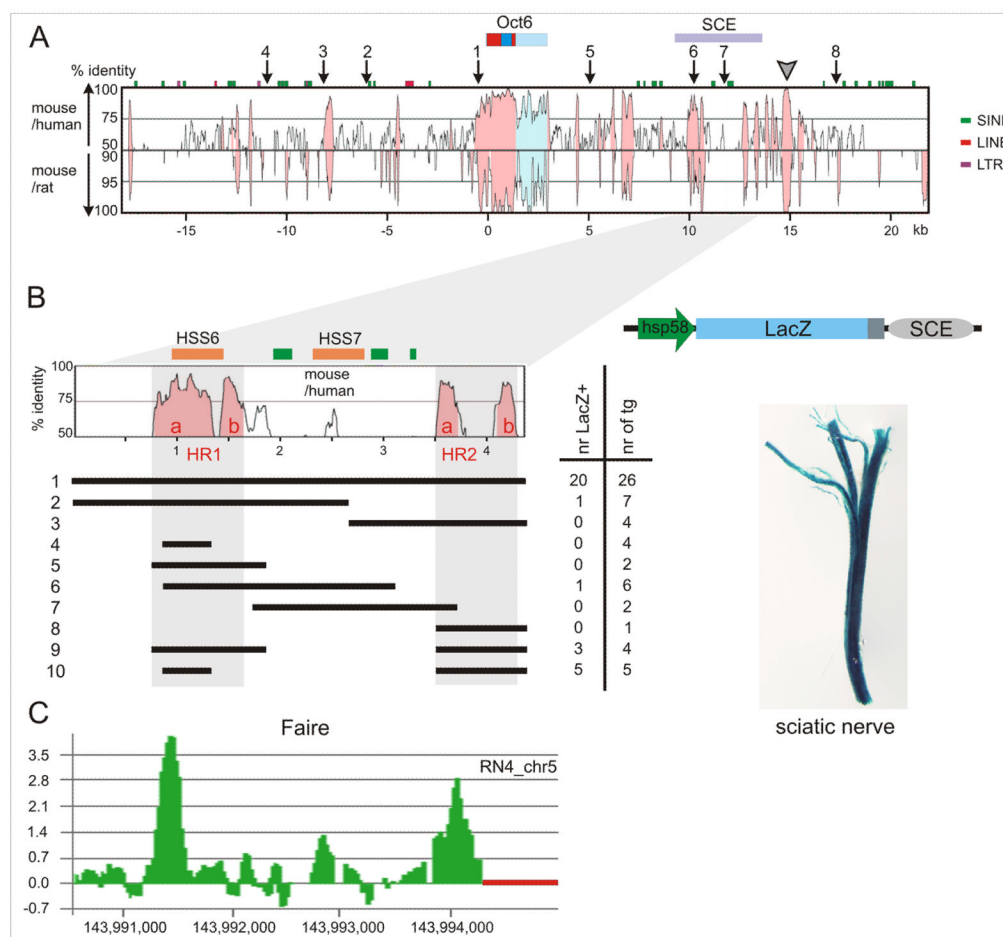
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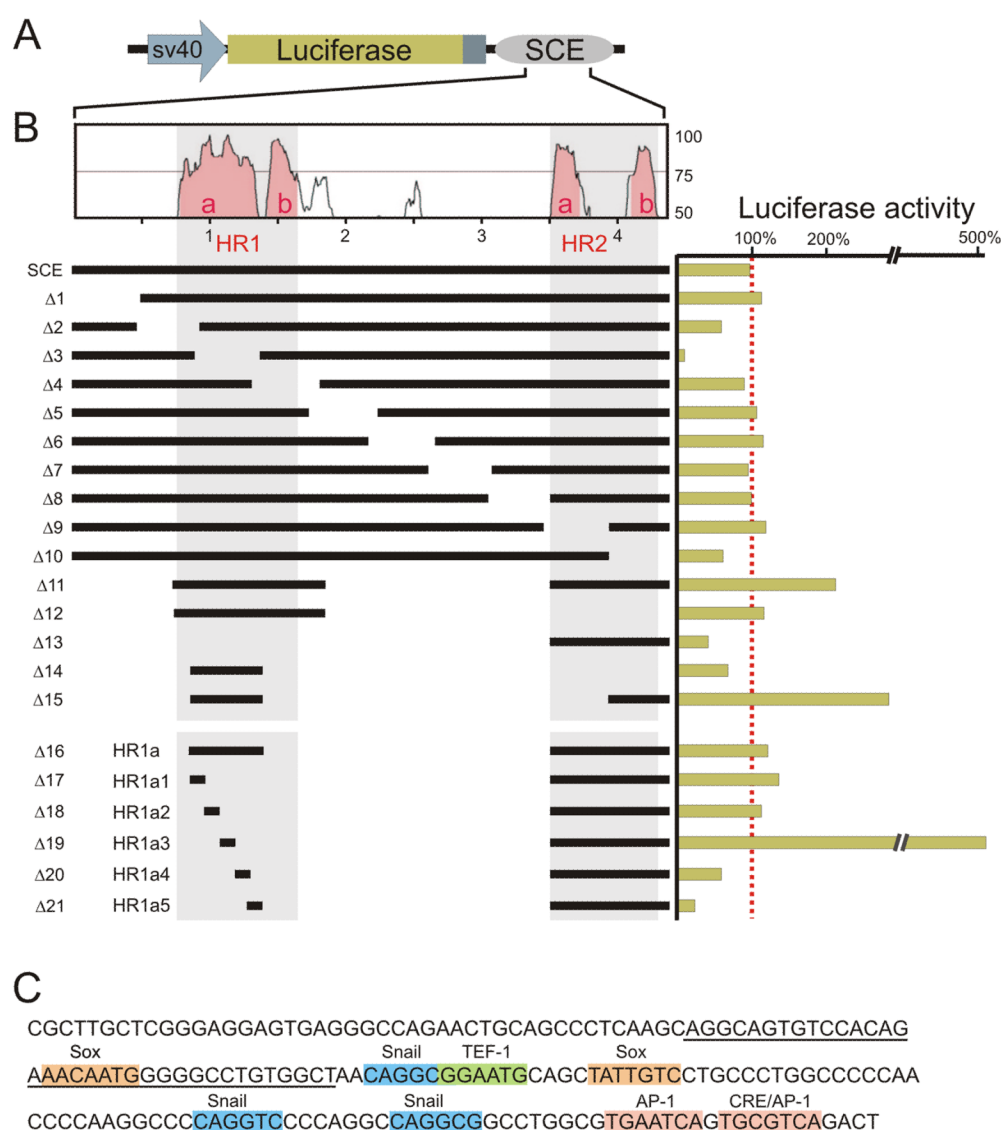
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**Figure 1. Comparative genomic and functional analysis of the mouse Oct6 locus**

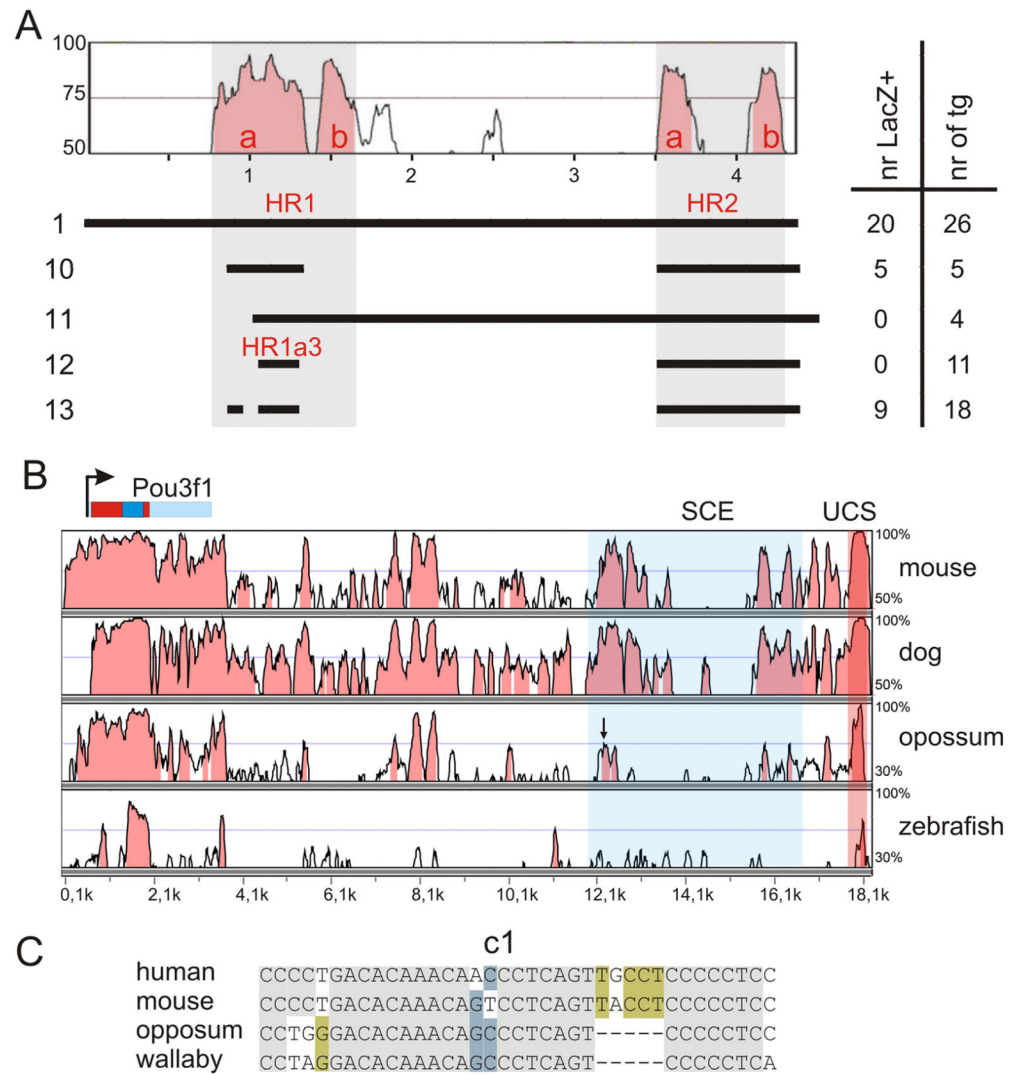
(A) Vista alignment of the mouse, rat and human Oct6 locus spanning approximately 50kb. The position of the Oct6 transcription unit and the SCE are indicated above the plot. The Oct6 open reading frame is in red with the POU domain in dark blue and the 3' untranslated region in light blue. The grey arrowhead marks the position of the ultra-conserved sequence and numbered arrows the position of the previously mapped DNaseI hypersensitive sites (Mandemakers et al., 2000). Repeat sequences present in the mouse genome are indicated. Note that the homology threshold has been adjusted to 90% for the rat sequence.

(B) The enlarged section of the SCE region shows the four regions of strong homology HR1a and b and HR2a and b between mouse and human sequences. The approximate position of HSS6 and 7 as well as the short repeat sequences (in green) are indicated. The black lines represent the segments of the SCE that were cloned behind the hsp68LacZ reporter construct. Individual constructs were tested for activity in transgenic mice and the number of transgenic founders (nr of tg) and those that express the hsp68LacZ in the peripheral nerves (nr LacZ<sup>+</sup>) are indicated. A dissected sciatic nerve of a P2 mouse transgenic for construct 9 is shown as a typical example. (C) The FAIRE plot of the rat Oct6 SCE is shown at the same scale as the Vista plot in B to allow a direct visual comparison of the two plots. The coordinates of the rat genome sequence (RN4) are indicated. The red bar indicates sequences not represented on the custom made micro array used in this experiment.

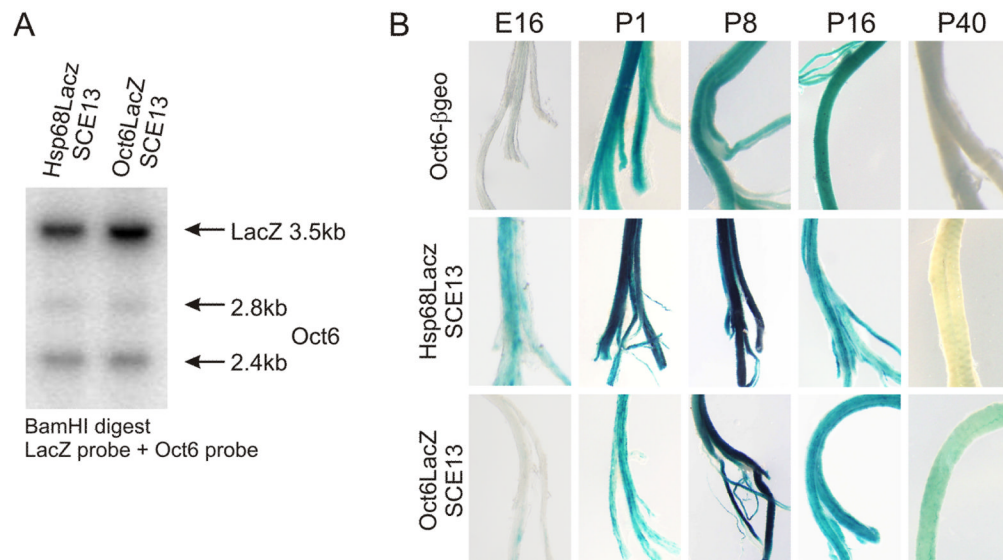


**Figure 2. Dissection of Oct6 SCE functional elements in differentiating Schwann cell cultures**  
 (A) SCE constructs were cloned behind the minimal SV40 promoter-driven Luciferase cassette (pGL3) and transfected in rat Schwann cells. (B) The SCE constructs tested are represented with a black line and aligned with the Vista plot. Luciferase activity of each construct is expressed as a percentage of the activity of the full SCE in these experiments. All transfections were performed in triplicate. (C) The sequence of HR1a3, which has strong enhancer activity in the luciferase assay, shows potential binding sites for transcription factors known to be involved in Schwann cell biology. These potential binding sequences were identified using the phylogenetic footprinting program ConSite. The sequence of the c2 element, containing the consensus Sox binding site, is underlined.



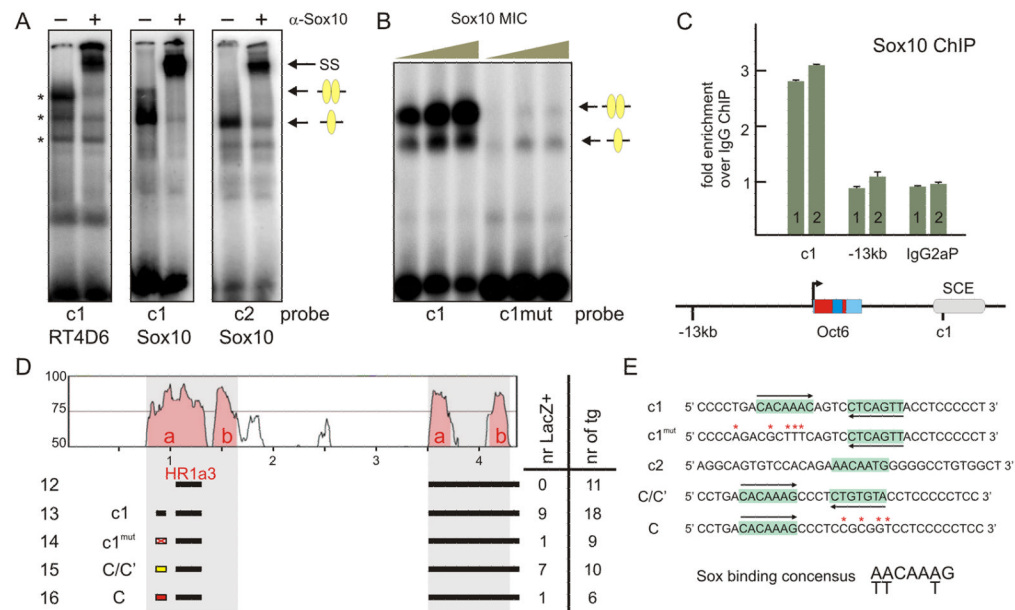


**Figure 3. Functional analysis of SCE elements in transgenic mice and comparative genomics**  
(A) Schematic depiction of the SCE constructs tested in transgenic mice. The SCE constructs are represented by black lines aligned with the Vista plot of the mouse SCE to visualize the presence or absence of conserved sequences in the various constructs. The total number of transgenic animals (nr of tg) and the number of animals that express the transgene in neonatal peripheral nerves (nr LacZ<sup>+</sup>) is indicated. All animals were analysed as founders.  
(B) Vista alignment of human SCE sequence with mouse, dog (*Canis familiaris*), opossum (*Monodelphis domestica*) and zebrafish (*Danio rerio*). Note that the lower homology threshold has been set to 30% for opossum and zebrafish to allow for the visualization of more diverged homologous regions. The position of core element c1 in the Vista plot of opossum is indicated by an arrow.  
(C) Alignment of core element c1 in the SCE of human, mouse, opossum and wallaby (*Macropus eugenii*). Hundred percent homologous nucleotides are boxed in black, 75% homology in light blue and 50% homology in ochre.



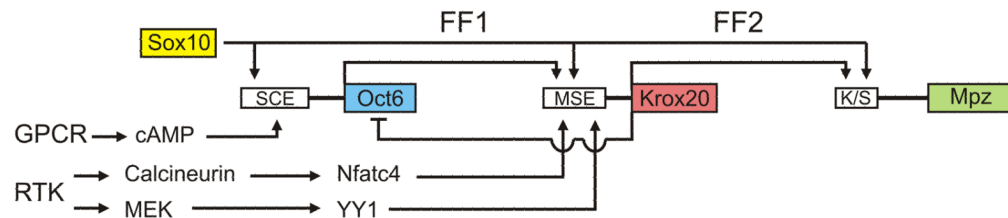
**Figure 4. Developmental control of Schwann cell specific expression through SCE13**

(A) Southern blot of genomic DNA of the two transgenic lines analysed here shows that they carry a comparable number of transgenes. The transgene is detected with a LacZ probe that hybridizes to a 3.5kb BamHI restriction fragment derived from the transgenic construct. The Oct6 locus is detected with a probe that hybridizes with two fragments of 2.8 and 2.4kb in length. The equal intensity of these endogenous band shows that equal amounts of DNA were loaded in both lanes. (B) Transgenic animals carrying a LacZ reporter gene driven by either the Oct6 promoter or the Hsp68 promoter and controlled by SCE construct 13, Oct6LacZSCE13 and Hsp68LacZSCE13 respectively, were stained for  $\beta$ -galactosidase activity in Schwann cells of the sciatic nerve at different stages of pre- and postnatal development. Developmental expression of  $\beta$ -galactosidase activity in nerves of the two transgenic lines was compared to the  $\beta$ -galactosidase activity in Oct6  $\beta$ -galactosidase-neomycin (Oct6- $\beta$ geo) heterozygous knock in animals (Jaegle et al., 1996; Mandemakers et al., 2000).



**Figure 5. Biochemical and functional analysis of core element c1**

(A) EMSA with radiolabelled doublestranded oligonucleotides c1 and c2. The sequence of these probes is depicted in panel E. Oligonucleotide probes were incubated with nuclear extracts from RT4D6 cells or from HEK293 cells over-expressing Sox10 (labelled Sox10), in the presence (+) or absence (-) of Sox10 antibodies, as indicated. The three major DNA-protein complexes formed on c1 with RT4D6 nuclear proteins are marked with an asterisk. The Sox10 monomeric and dimeric complexes are indicated with arrows. (B) EMSA with radiolabelled doublestranded oligonucleotide c1 and c1<sup>mut</sup>. Oligonucleotide probes were incubated with increasing amounts of nuclear extracts from HEK293 cells expressing Sox10 MIC. (C) ChIP of Oct6 SCE sequences using Sox10 antibodies. Chromatin of RT4D6 cells was precipitated with Sox10 or rabbit IgG antibodies. The non-specific rabbit IgG antibodies served as a control. Quantitative PCR on immune-precipitated and purified DNA using primers for the c1 region (c1), a region 13kb upstream of the Oct6 promoter (-13kb) and a region associated with the IgG2a promoter (IgG2aP) was performed in triplo and was used to determine the relative enrichment for these loci by Sox10 over IgG immune-precipitation. The experiment was performed twice. Data from both experiments are presented (1 and 2). (D) Functional analysis of Sox binding sites in element c1. The *in vivo* activity of the various SCE constructs was assessed in new born founder animals. The number of transgenic animals (nr of tg) and the number of animals that express LacZ in the peripheral nerves (nr LacZ<sup>+</sup>) is indicated. Constructs 14, 15 and 16 were created from construct 13 by replacing the c1 sequence for that of c1<sup>mut</sup>, C/C', and C respectively. (E) Sequence of c1 and related elements and its various mutant derivatives. Red asterisks indicate the nucleotides mutated in c1<sup>mut</sup>. The position and orientation of the Sox10 binding sites is indicated by green boxes and arrows. The C/C' and C sequence is derived from the *Mpz* promoter (Peirano et al., 2000). Differences between C/C' and C are indicated with red asterisks. The consensus Sox DNA binding motif was derived by Harley and colleagues (Harley et al., 1994)



**Figure 6.**

The figure provides a schematic representation of the gene regulatory network that governs the transition from a premyelinating Schwann cell to a myelinating cell and incorporates findings described in this study. Different signalling pathways converge on the regulatory elements in this network and cooperate with Sox10 to control Oct6, Krox20 and myelin gene expression (Svaren and Meijer, 2008). The Sox10 dependent activation of Oct6 and subsequent synergistic activation of Krox20 defines a feed-forward (FF) regulatory module. Sox10 then cooperates with Krox20 to control expression of myelin related genes, through closely spaced Krox/Sox (K/S) binding elements, defining a second feed-forward loop. These FF loops serve to time and amplify the onset of myelination in the PNS. For further details see text.